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14. ABSTRACT

Applying a functional strategy, starting with the transfer of an intact chromosome 16, we first mapped the position of a cell senescence gene, SEN16, at 16q24.3. Precise positional information led to the identification of YAC and BAC clones that restores normal growth pattern and senescence in breast tumor cells. cDNAs corresponding to the transcripts, encoded from the genomic region of the BAC, were isolated and sequenced. The sequence information of known cDNAs was used to isolate full length cDNA clone, encoded from SEN16 locus. Individual cDNAs were cloned into mammalian cell expression vectors and tested for the restoration of senescence. Ectopic expression of one of the cDNAs, in tumor cell lines led to terminal growth arrest and senescence. Analysis of breast tumor and other tumor cell lines revealed genomic rearrangements at SEN16 locus, as well as loss of expression of the complementing cDNA. These results suggest that the cDNA that we have cloned in deed is tumor suppressor gene. In future studies, we will clone the gene in an inducible vector for further analysis. An inducible vector will provide the benefit of controlled expression of the gene in tumor cells to examine the effect on the cell cycle. In silico analysis or the cDNA sequence revealed that predicted protein is a part of an ubiquitin - ligase complex that may function in protein degradative pathway. Malfunctioning of these pathways may contribute to the deregulation of cell cycle, leading to the development of cancer. Further characterization of SEN16, for the identification of signaling pathways will afford insight into deregulation of cell growth and senescence. We have already initiated the experiments, using yeast two hybrid system to identify interacting proteins. At the end we anticipate that our studies will lead to the identification of new diagnostic markers, which eventually may lead to the development of new diagnostic and therapeutic strategies.

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)

Breast Cancer, Cell senescence gene, SEN16, Tumor suppressor gene , functional analysis

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A. Introduction (Adapted from the original proposal)

Normal diploid mammalian cells display a limited proliferative life span in culture (1-3). At the end of the proliferative phase, cells enter a state of irreversible post mitotic growth arrest called senescence (1-3). Mouse hyperplastic alveolar nodule (HAN) cells can be passaged indefinitely from animal to animal by implanting in mouse mammary fat pads and are at high risk for tumor development. In contrast, normal mammary epithelial cells form normal ductal tree and cannot be passaged for more than 3-5 times (4). Spontaneous escape from senescence can occur in most rodent cells (5) but has never been reported in human cells. Normal human cells can be transformed by exposure to radiations (6), oncogenic viruses (7) or chemical carcinogens (8), but additional genetic alterations are required to acquire indefinite proliferation (9, 10). Immortalization, therefore, appears to increase the susceptibility to malignant transformation, while cell senescence protects the organism against the consequences of deregulated cell growth (11).

Although a number of molecular differences between immortal and senescent cells have been observed, the changes in gene expression which induce cellular senescence and which result from the onset of senescent program are not known (reviewed in 12). While abrogation of p53 and pRb expression allows normal human fibroblasts to grow for an additional 10-20 doublings, inhibitors of cyclin dependent protein kinases (CDKs), p21 and p16, are over expressed in senescent cells. Serum stimulated senescent cells remain at the G1 restriction point and fail to induce G1 genes but are resistant to apoptosis (13). The well documented progressive loss of telomere length due to diminished telomerase activity in normal cells, but not in immortal cell lines, is an attractive candidate for a “molecular clock” that limits cell proliferation to a finite number of replications (14, 15). However, this correlation is not absolute (16).

In somatic cell hybrids between normal and immortal cells, cellular senescence is expressed as a dominant phenotype over indefinite proliferation (17, 18). By the transfer of individual human chromosomes into a variety of immortal cell lines, cell senescence genes have been mapped on at least ten different chromosomes (19-25). Although several different chromosomes were found to restore normal cell growth and senescence in breast cancer cells, we chose to focus on chromosome 16, because abnormalities in 16q have been repeatedly documented in breast as well as in other tumors (26). Successful positional cloning strategies have been devised to isolate disease genes using linkage analysis and chromosome rearrangements to identify the locus. However, these strategies cannot be applied to the cloning of cellular senescence genes because these cannot be identified in LOH studies or tracked by linkage analysis. To overcome these difficulties, we have devised a ‘Functional-Positional Cloning’ approach (27-29). Starting with intact chromosomes, using functional criteria to track the gene, we mapped a cell senescence gene, SEN16, within 2-3cM genetic interval at 16q24.3 (29). We have now isolated a BAC clone carrying SEN16 gene. Here we propose to clone cell senescence gene, SEN16 and study its role in the development of breast cancer.

B. Body

Since our previous report was almost a year late, experiments performed during the time period covered for this report was included in the last report. However, the project was continued with the funds conserved in the budget. Here we report the results of the experiments performed following the last report.

I. The specific aim of the original proposal:

Aim 1: a. Isolation of expressed sequences for BAC 346J21
b. Identification of candidate cDNA clones

Aim 2: a. Isolation of full-length cDNA clones
b. Functional analysis of full-length cDNA clones.
c. Structural analysis of Senescence Gene
Aim 3: Analysis for Mutational Inactivation of SEN16
Aim 4: Analysis for the Expression of SEN16 in immortal breast tumor & normal cells.

II. Physical and Transcript map of SEN16 locus

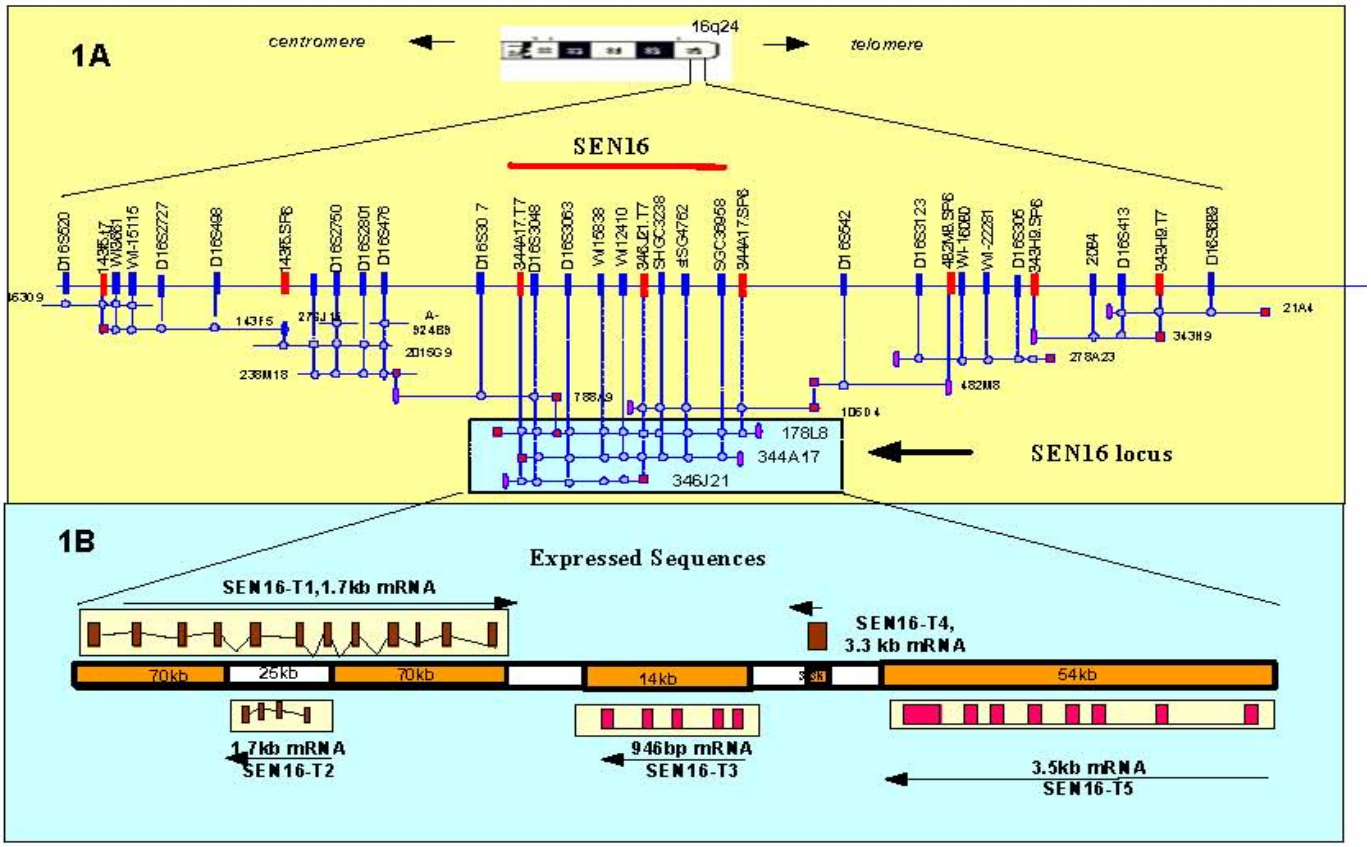

Fig.1 Physical and transcript map of SEN16 locus

A. Contig of 15 BAC clones along with mapped markers.

B. cDNAs corresponding to the transcribed sequences from SEN16 locus. These cDNA are assembled from sequence of partial EST clones, sequence of RT-PCR results and database search. Since all cDNAs are not characterized as yet, each cDNA is designated as transcriptional unit named as SEN16T1, SEN16T2, SEN16T3, SEN16T4 and SEN16T5.

We have constructed a transcript map for SEN16 region and cloned a number of full length cDNAs, encoded from SEN16 locus (Figs. 1B and 2). We temporarily named these cDNAs as transcription units SEN16T1, SEN16T2, SEN16T3, SEN16T4 and SEN16T5 (**T** stands for transcript).

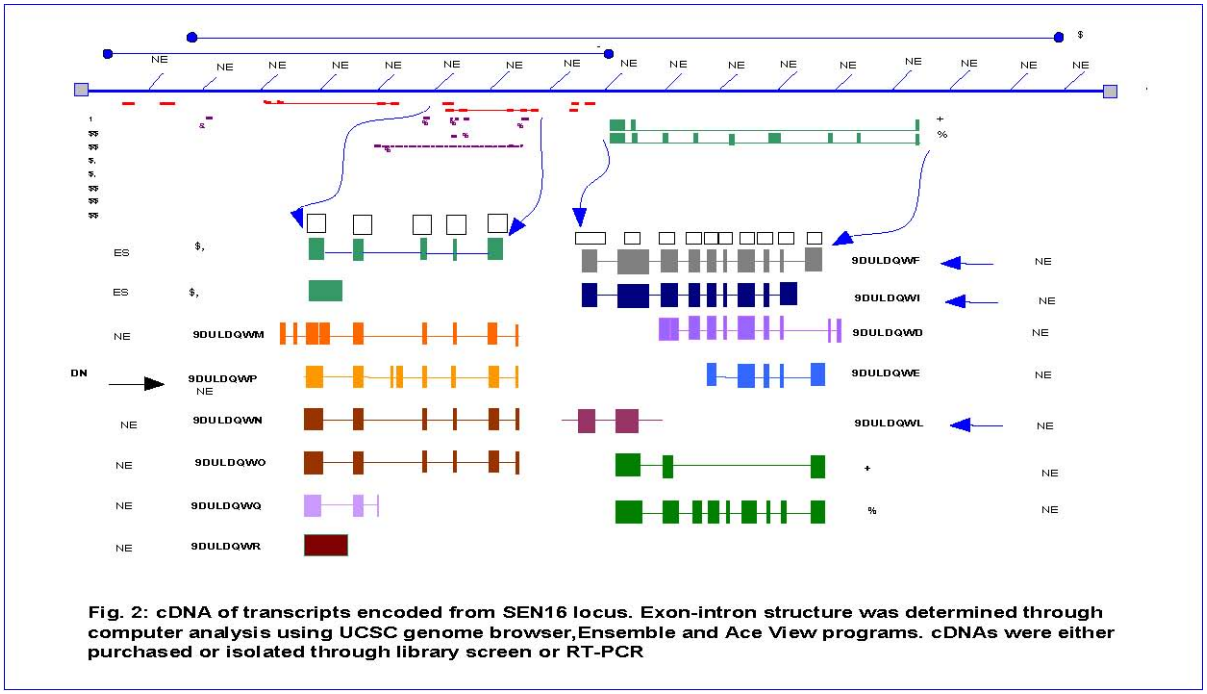

Fig. 2: cDNA of transcripts encoded from SEN16 locus. Exon-intron structure was determined through computer analysis using UCSC genome browser,Ensemble and Ace View programs. cDNAs were either purchased or isolated through library screen or RT-PCR

While SEN16T1 is a 1.7Kb transcript that contains 5 exons and is encoded from the positive strand (i.e. 5' centromere ' to 3' telomere), SEN16T2 is 1.7 Kb in size comprised of 4 exons encoded from the same region

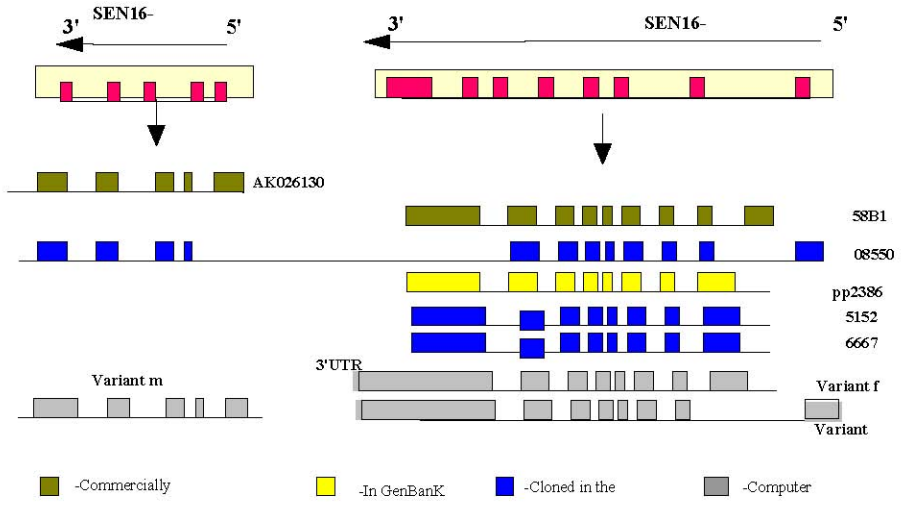

Fig.3 Full-length cDNA open reading frames isolated by RT-PCR for regions SEN16T3 and SEN16T5 show the presence of splice variants encoded from these regions.

but from the negative strand of the genomic DNA. SEN16T3 is a 0.95 Kb transcription unit with multiple splice forms, each comprised of 5 exons, expressed from the negative strand. SEN16T4 is a 3.0 Kb single intron less transcript that also comes from the negative strand. Several different splice variants of SEN16T5, each comprised of different exons are expressed from the negative strand in normal and tumor cells. Some of the splice variant forms of SEN16T5 are different in tumor cells than those in normal cells. Expression of each of these transcripts has been experimentally verified by RT-PCR and sequencing analysis. The open reading frame, translation initiation and stop codon for each of the transcripts (SEN16T1, SEN16T4 and SEN16T5) were identified by computer analyses.

PCR primers designed from the ends of each transcript were used to search for intervening linking transcript segments. Interestingly, our investigations revealed that SEN16T3 and SEN16T5 are parts of a single gene and expressed as two independent genes as well. Thus making it as three different genes (Fig. 3). Each of the proteins encoded from these three genes may have a different function. We have identified 9 different full-length ORF for the transcripts encoded from SEN16T5/T3 regions (Fig. 3**).** These appear to be a highly complex set of genes, with yet unknown number of splice variants.

III. Expression in different tissues

Multi-tissue Northern blot analysis, using cDNA probes, revealed the expression of transcripts varying from 1kb to 6kb in size which are differentially expressed in different tissues (Data presented in previous report). Also isoform detected in breast cancer cells were different than those in normal cells (Fig 3). We are continuing these investigations to further define the structure of different genes located at SEN16 locus.

IV. Cloning of full-length cDNAs

Five different transcription units (Fig. 1B) were identified from computer analysis of human genomic sequences and from the sequence of partial cDNAs cloned by others and us. Three of the putative genes, located

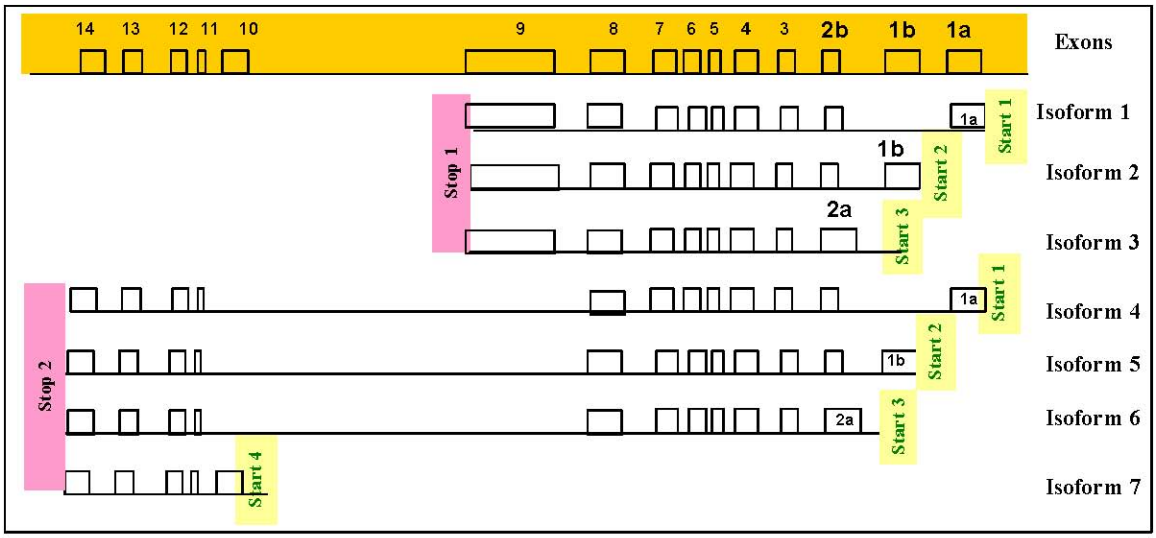

Fig. 4. Isoforms encoded from SEN16T5/T3 transcription units utilizing different start and stop sites, verified by RT-PCR.
Isoform 1 is predicted and utilizes transcription start-1 and stop-1.
Isoform 2 *(Clone 58B12)* represents transcription from start-2 to stop-1, and includes exons 1b through 9.
Isoform 3 *(Clone p2386, 5152, and 6667)* represent transcription from start-3 to stop-1, including exons 2a through 9.
Clone **5152** contains a variant (shortened) exon 8, and clone **6667** includes a unique small exon between 7 and 8.
Isoform 4 *(Clone 08550)* represents read-through transcription from start-1 to stop-2, and includes all exons except 9 and 10, which lacks 3' and 5' splice sites, respectively.
Isoform 5 is predicted and utilized transcription start-2 and stop-2.

in the predicted region of SEN16, were examined in greater detail for ORF and splice variation (Figs.1B and 4). The cDNA clones originating from the region 16T5/16T3, each have 5' ends located at one of the four putative translation start sites, and 3' ends located at one of the two putative stop sites (Fig. 4). Sequences included in these cDNAs mostly fall into 14 exons; an additional exon (between 7 and 8) and a truncated exon 8 (not shown) each occur once, in separate clones. Presence of four start sites in combination with two stop sites will predict the existence of seven possible transcript isoforms for SEN16T5/T3 (Fig.4). While isoforms 2, 3, 4 and 7 have been confirmed

by cDNA cloning (Fig. 4), isoforms 1, 5 and 6 are predicted.

Four different full-length cDNAs, representing different variants of two genes (SEN16T3 and SEN16T5, Fig. 3) and one full-length cDNA representing a third gene (SEN16T1, Fig. 1B) were isolated by RT-PCR. Each of the genes have been cloned into a mammalian cell expression vector and verified for sequence integrity of the coding region. Experiments to identify and clone additional isoforms are in progress.

V. Functional Analysis of full length cDNAs

Five different transcription units (Fig. 1B) were identified from computer analysis of human genomic sequences and from the sequence of partial cDNAs cloned by others and us. RT-PCR with primers located at farthest end of the transcripts identified 7 full length ORF (Fig. 5). Four of the full length cDNAs, 2P1, E, ICI

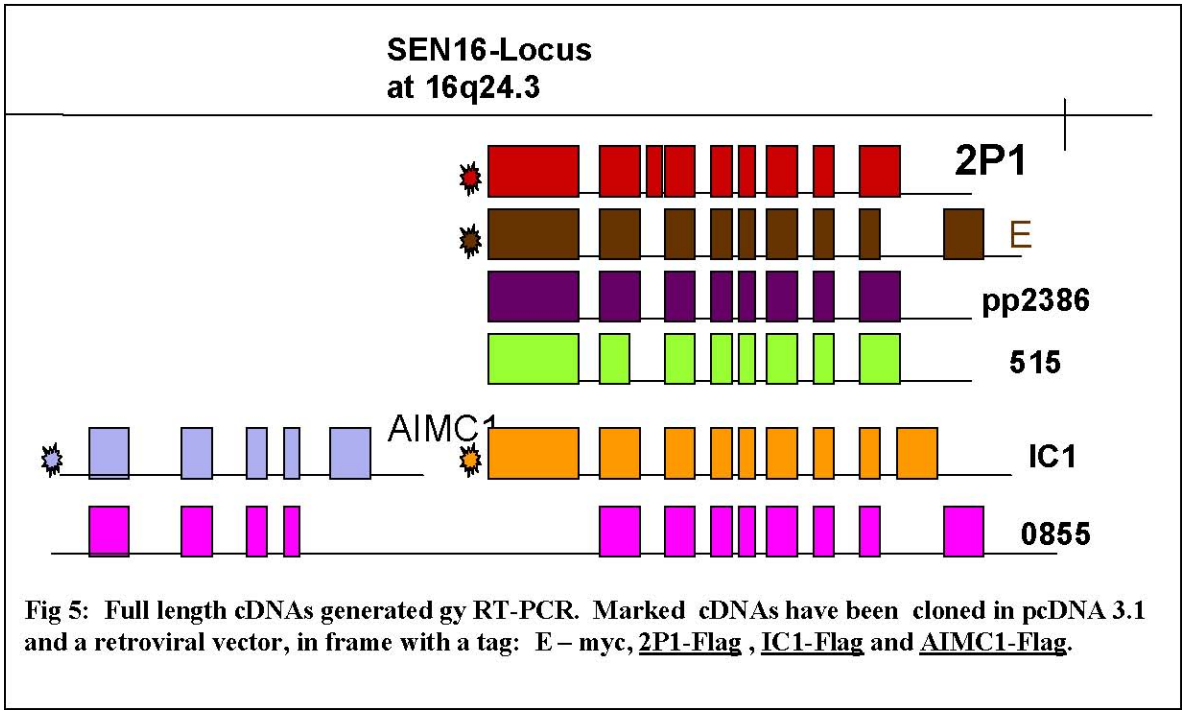

Fig 5: Full length cDNAs generated gy RT-PCR. Marked cDNAs have been cloned in pcDNA 3.1 and a retroviral vector, in frame with a tag: E – myc, 2P1-Flag , IC1-Flag and AIMC1-Flag.

and AIMC1 (Fig. 5), were cloned into a mammalian cell expression vector pcDNA3., in frame with FLAG or myc tag. Cloned genes were verified for sequence integrity of the coding region and the tag. Plasmid construct containing AIMC1 was transferred into mammary tumor cell lines, MCF.7, MDA-MB468 and LA.7. G418 resistant gene transfer colonies were either followed in the parent plates or isolated individually. All colonies were examined at regular intervals to assess colony and cell morphology and growth characteristics. Following ectopic expression of AIMC1 in MDAMB468 cells, two types of colonies
i.e. senescent and immortal were recovered. Cells in senescent colonies displayed a progressively diminished growth rate leading to complete growth arrest. At this stage cells attached to the surface were stained for senescence associated β-gal (SA β-gal) activity (Fig.6). Senescent cells remain attached to the plates for long periods of time. To the extent that one of the colony was maintained in culture for longer than 6 month period.

Senescent colonies displayed characteristic peri-nuclear staining showing the expression of SAβ-gal activity (Fig. 6). In contrast, immortal colonies were indistinguishable from the parental cells, which multiplied indefinitely and were negative for SA β-gal activity. PCR analysis of DNA isolated from individual colonies revealed that immortal colonies did not contain intact cDNA, but carried the *neo* marker. Loss of the cloned fragment could occur during the process of transfection or integration into the cell genome. In comparison, all senescent colonies analyzed, contained intact cDNA. Due to the limited number of cells present in senescent colonies, analysis for cell cycle parameters could not be performed. The number of colonies, recovered in vector control v/s AIMC1 gene transfer, was at least 50 fold more. These data suggested that AIMC1 gene, expressed from a strong CMV

promoter, leads to immediate growth arrest and senescence giving reduced number of viable colonies.

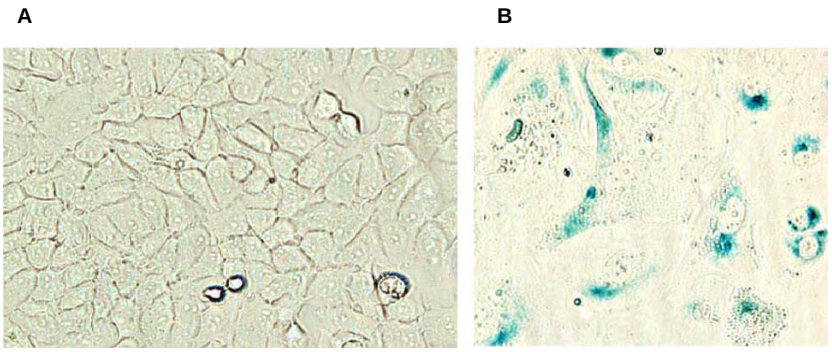

Fig. 6. Photomicrographs of cells stained for Senescence Associated b-gal Activity (SA-b-gal). (A) MCF. 7 cells showing no detectable SA-b-gal activity. (B) A gene transfer growth arrest clone of MCF.7 containing cDNA clone 5152, showing characteristics of senescent cells and perinuclear β-gal staining.

The experiment performed on the transfer of AIMC1 into MCF.7, T47-D and LA7 cells were inconclusive. For each of these cell lines 10 independent colonies were isolated and preserved for analysis. However, introduction of pcDNA3-515 into MCF.7 cells led to the terminal growth arrest. In this case colonies were positive for SAβ-gal activity (Fig. 6).These experiments suggest that both cDNAs can induce senescence in breast tumor cell lines. These studies are currently being repeated, using retroviral vector system to further verify our findings.

VI. Difficulties Encountered in these Experiments:

The results of the above experiments suggest that over expression of AIMC1 cDNA, driven by a strong generic promoter, limits the proliferation potential of the cell and induces senescence at an early stage. Thus, using pcDNA3.1 vector, it may be difficult to obtain an adequate cell population of gene transfer clones for further analyses. Also, tag protein FLAG used for detection of the AIMC1 expression binds to a non specific product in size range of expected distinguish the AIMC1 product from the non specific binding.

We have now cloned these genes into a retroviral vector (PQCXIP) in frame with EGFP tag. This will allow us to follow the gene expression by immunofluorescence and western blot analysis. A retroviral vector provides highly efficient system for rapid analysis of all different cDNAs in multiple cell lines. Although retroviral vector systems with CMV promoter will be useful for initial screening of a large number cell lines for the effect of each isoform, it may be difficult to obtain an adequate cell population of a gene transfer clone for further analyses.

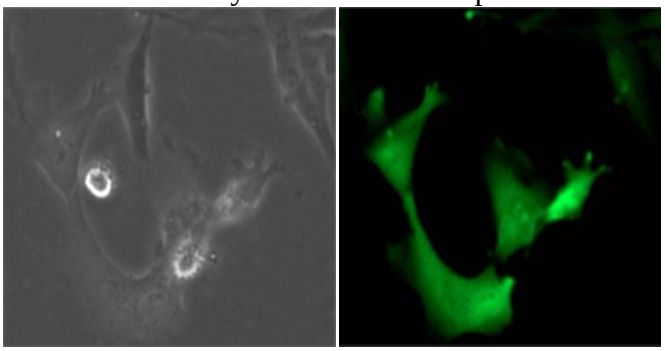

Fig. 7. A clone of MCF.7 cells, expressing RXR and VgEcR recectors, was transfected with pIEGFP and induced with Muristerone A (10uM) for 48h, to test for the induction of the cloned gene. (A) Phase contrast and (B) Fluorescent photomicrographs showing the induction.

In order to get around the problem of over expression induced instant growth arrest, we will clone each of the genes in a vector system with an inducible promoter. In this case, we will have the advantage of inducing the message in the cells when required. We are using a retroviral based vector system with ecdysone-inducible promoter from Stratagene. This system is comprised of two vectors. One of the vectors pFB-ERV, carries genes for retinoid X receptor (RXR), ecdysone receptors (VgEcR) and *neo* driven by CMV promoter as a single transcript. The second vector, pCFB-EGSH, which is used for cloning the gene of interest, carries ecdysone glucocorticoid response element (E/GRE) sites, located upstream to minimal heat shock (mHSP) promoter along with *hyg* gene for selection in mammalian cells. Each vector will be propagated in an appropriate packaging cell line and introduced into the tester cell lines through viral infection.

In the presence of inducer ecdysone or its analogs Muristerone A or Ponasterone A, RXR and VgEcR

form dimers. The dimerized complex acts as a transcription factor, binds to E/GRE sites, located in the second vector upstream to the mHSP promoter, and induces the expression of the cloned gene. In the absence of the inducer, two receptors RXR and VgEcR cannot form dimers and fail to bind to E/GRE sites and the gene remains turned off. Since the system is dependent upon the expression of two receptors, it provides a non-leaky complete control of gene expression . We have already generated clonal tester cell lines, expressing RXR and VgEcR receptors for breast tumor cell lines SKBR3, T47D and MCF7 (Fig 7).

The preceding experiments will allow us to determine the optimum time period required for the onset of senescence, which will be used in later studies. After making an initial estimate of total population doublings (PDs) between gene induction and senescence, detailed experiments will be conducted at defined time intervals, to determine **i)** the proliferative capacity of transfected cell populations by growth curves**, ii)** BrdU incorporation to check DNA synthesis **iii)** flow cytometric analysis for DNA content and **iv)** telomerase activity and telomere length. For negative controls, cells transfected with an empty vector and an unrelated gene cloned into the same vector will be compared with the experimental clones.

VII. Identification of Mutations at SEN16 locus

a. Analysis of immortal cell lines for genomic deletions at SEN16 locus: Mutations resulting in a disease phenotype can be classified into two major categories: those that cause major changes in gene structure (large deletions, insertions, duplications and inversions) and those that cause only a minimal change in gene structure (single base changes as well as small deletions, inversions, duplications, or insertions). Southern blot hybridization and restriction mapping can easily identify mutations in the first category.

We used AI861895 as a molecular probe for detecting deletions or rearrangements in 22 immortal tumors cell lines derived from different tumors. Since we knew the genomic sequence of the region, the size of the expected hybridization bands can be precisely predicted**.** Any missing band or altered size of the bands would indicate genomic rearrangements**.** Our results revealed genomic rearrangements in three breast cancer cell lines, T47D, SKBR.3 and MCF.7at SEN16 locus. In addition PCR amplification for known markers, located at SEN16 locus, revealed homozygous deletions in some of the cell lines.

b. Analysis for the expression of cDNA sequences in immortal cell lines: 22 immortal cell lines, derived from different tumors or SV40 transformed human cells, were examined by RT-PCR for the loss of RNA transcripts for partial cDNAs from SEN16 locus. These data revealed the loss of expression of a clone AA906579 (representing SEN16T5 transcript) in MCF.7, a breast cancer cell line, and in cl39, an SV40 immortalized cell line. Chromosomal region 16q24.3 displays high incidence of LOH in many different cancers and has been under intense investigation for tumor suppressor genes in several different labs around the globe. The genomic rearrangements observed at the SEN16 locus, especially in the expressed sequences, in tumor cell lines, suggest a possible role for this region in the etiology of breast cancer.

VIII. Identification of Interacting proteins by Yeast Two Hybrid System:

Having identified a putative gene for cellular aging and senescence, we proceeded to identify the proteins that interact with the product of full length cDNAs. Yeast two-hybrid assay provides a simple method to identify protein-protein interactions and to define interacting domains of a protein. This system uses transcription activating and DNA binding domains of a well-characterized yeast transcription factor GAL4. In this system, gene for bait protein is expressed as fusion to GAL4 DNA binding domain (DBD), and cDNA of the search library is expressed as a fusion to GAL4 activation domain (AD,). Two cDNAs are brought together in a yeast diploid hybrid cell by mating host strains containing bait cDNA and library cDNA. When bait fusion protein and library fusion protein interact with each other in yeast diploid reporter strain, DBD and AD of GAL4 are brought closer to each other, which in turn activates the transcription of reporter genes (i.e ADE2, HIS3, lacZ and MEL1). Diploid yeast clones positive for reporter gene, are isolated by selection in minimal medium that only supports the growth, where reporter genes are expressed.

We used Matchmaker GAL4 Two-Hybrid vector System 3 from BD Biosciences-Clontech (PaloAlto,

CA). Three transcripts from SEN16 locus were cloned into pGBKT7 next to GAL4DBD and transformed into yeast strain AH109. Transformation mixture was plated on SD medium lacking Trp. Each of the transformant yeast clone was checked by western blotting to confirm the expression of fusion protein.

For screening, we used a pre-transformed human HeLa Matchmaker cDNA library from BD Biosciences. This library is constructed in pACT2 vector and transformed into yeast strain Y187. This vector contains LEU2 nutritional gene for selection on limiting synthetic medium and encodes for the fusion protein from cDNA - GAL4AD, driven by a constitutive ADH1 promoter. The yeast strain Y187, expressing library cDNA, was mated with AH109 that expresses SEN16 transcripts. Yeast diploids, expressing both interacting proteins, were isolated by selection on high stringency selection media (-Met, -Leu, -Trp, -His, -Ade, +Xgal), which support the growth of diploids expressing TRP1, LEU2, HIS3, ADE2, lacZ, MEL1 and GAL4, induced by the association of fusion proteins SEN16-GAL4 DBD and cDNA -GAL4 AD. Positive clones, isolated on to high stringent selection medium, were further verified for growth on high stringency selection medium and by β-gal assay. Selected colonies, which should contain pGBKT7/SEN16 construct and a pACT2/cDNA construct from the library, were then transferred to a master plate. We have isolated a total of 400 clones for further analysis. Clones containing more than one cDNAs, were discarded following PCR analysis of the cell lysates , using pACT2 vector specific primers. We are in the process of rescuing the inserts from these clones by PCR and clone into a plasmid vector for sequencing. The nucleotide sequence of different clones will be compared with each other to identify clones with unique cDNA inserts, and to eliminate identical clones. The sequence of unique clones will be compared with sequences in the GenBank, EMBL and other human genome database. These data will identify corresponding gene and will also reveal if different cDNA inserts, in the library, belong to same or different genes. Following initial identification, interactions between SEN16 and interacting proteins in the selected clones will be confirmed by immuno-precipitation with anti-myc or anti-HA, and by Western hybridization.

C. Key Research Accomplishments

1. Applying a functional approach, starting with an intact chromosome, we progressively reduced the genomic region carrying SEN16 to a 185 Kb DNA segment carried in BAC clone 346J21, which restores senescence in the breast tumor cell lines MCF.7 and LA7.
2. We identified candidate cDNAs encoded from the genomic sequence in BAC 346J21.
3. While multiple transcript variants are encoded for candidate cDNAs in normal and tumor cells, the variants identified in immortal cells are different from those in normal cells.
4. We have cloned five different full-length cDNAs, representing three novel genes located at the SEN16 locus, in mammalian cell expression vectors.
5. Full length cDNA ORPs were cloned in two different mammalian cell expression vectors, pCDA3.1 and a retroviral vector
6. Ectopic expression of one of the cDNA restore ed senescence in one of the breast cancer cell line (MDA-MB-468). However, experiments with MCF.7 and T47D cells are currently being repeated.
7. We have cloned the cDNA in a retroviral vector in frame with GFP. In future experiments these clones will be tested for the effect on the growth of breast tumor cell lines.
8. We have established retroviral based Ecdysone inducible expression system for further analysis of the cloned genes.
9. We have initiated experiments to identify proteins associated with SEN16 gene expression.These
10. Protein database search predicted the presence of F-box motif in two of the transcripts. Proteins containing F-box motif are known to interact with cell cycle proteins e.g. G1-Cyclins, cylin-cdk inhibitors and transcription factors.

The results of our experiments support that we indeed may already have identified a cDNA representing SEN16 gene. Future experiments are directed to identify signaling pathways associated with the expression of this gene.

D. Reportable Outcomes

Publications:

1. Reddy DE, Sandhu AK, DeRiel JK, Athwal RS and Kaur GP (1999) Identification of a gene at 16q24.3 that restores cellular senescence in immortal mammary tumor cells. Oncogene 18:5100-5107
2. Reddy DE, Keck CL, Popescu N, Athwal RS and Kaur GP (2000) Identification of a YAC from 16q24 carrying a senescence gene for breast cancer cells. Oncogene 19: 217-222.
3. Kaur, G.P.; Reddy, D.E.; Zimonjin, D.B.; deRiel, J.K. and Athwal, R.S. (2005) Functional identification of a BAC clone from 16q24 carrying a senescence gene SEN16 for breast cancer cells. Oncogene 24, 47-54.

Manuscripts in Preparation:

Lee Y and Kaur GP. Identification of SEN16 gene transcripts from 16q24 by expression analysis

Abstracts of Presentations:

1 Kaur G, Athwal RS and Kaur GP (2000) A functional-positional approach towards cloning a cell senescence gene at 16q24.3. Proceedings of 91st Annual AACR meeting, San Francisco, CA.

2 Kaur GP and Athwal RS (2002) Isolation of expressed sequences from a genomic segment carrying senescence gene (SEN16) for breast tumor cells. Amer. J. Hum. Genet. 71 (supplement): #419.

3 Kaur GP and Athwal RS (2003) Cloning of candidate cDNAs for a novel senescence gene in breast tumor cells. Proceedings of 94th Annual AACR meeting, Washington,DC.

E. Conclusion

By functional screening, we had previously identified an 85 kb BAC clone that carries SEN16 gene. A number of cDNA clones, corresponding to transcripts encoded from the SEN16 locus, were identified by human genome database search. Partial cDNA clones, obtained from commercial sources, were sequenced and verified for their location on the complementing BAC clone. The sequences of partial cDNA clones were compared with each other to identify overlapping clones. Additional cDNAs, spanning known partial cDNA clones, were isolated by RT-PCR with primers designed from the ends of partial cDNA clones. These data allowed us to assemble five different full-length cDNA clones representing three novel genes, located at the SEN16 locus. Expression analysis for two of the cDNAs revealed the presence of multiple transcript isoforms in normal and tumor cells. However, isoforms encoded in tumor cells are different from those in normal epithelial cells. In addition, DNA rearrangements at the SEN16 locus have been detected in some breast tumor cell lines. In preliminary studies, ecotopic expression of one of the cDNA clones inhibits growth in MCF, 7 cells and induces senescence. These results point to a role of SEN16 in cell growth and senescence. We have essentially accomplished all the aims as proposed in original proposal, and are continuing further to analyze tumor tissues for mutational spectrum and to investigate signaling pathways.

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Functional identification of a BAC clone from 16q24 carrying a senescence gene SEN16 for breast cancer cells

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We have identified an 85 kb BAC clone, 346J21, that carries a cell senescence gene (SEN16), previously mapped to 16q24.3. Transfer and retention of 346J21 in breast cancer cell lines leads to growth arrest after 8–10 cell doublings, accompanied by the appearance of characteristic senescent cell morphology and senescence-associated acid β-galactosidase activity. Loss of transferred BAC results in reversion to the immortal growth phenotype of the parental cancer cell lines. BAC 346J21 restores senescence in the human breast cancer cell lines, MCF.7 and MDA-MB468, and the rat mammary tumor cell line LA7, but not in the human glioblastoma cell line T98G. We postulate that inactivation of both copies of SEN16 is required for the immortalization of breast epithelial cells at an early stage of tumorigenesis. Positional mapping of 346J21 shows that SEN16 is distinct from other candidate tumor suppressor genes reported at 16q24.
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Introduction

Normal diploid human cells undergo replicative senescence after a finite number of divisions in culture (Hayflick and Moorhead, 1961; Hayflick, 1974). In contrast, cells cultured from many tumors can proliferate indefinitely. Cellular senescence is a genetically controlled dominant program and is characterized by distinct alterations in cell phenotypes. Senescence-related constraints on cell division are postulated as anticancer and escape from senescence is an important step in neoplastic transformation (Campisi, 2000, 2001).

The dominant nature of cellular senescence has provided an opportunity to identify senescence loci by microcell transfer of individual human chromosomes into immortal cell lines (Ohmura *et al*., 1995; Bertram *et al*., 1999; Tominaga *et al*., 2002). Applying this approach, we have previously identified cell senescence loci on human chromosomes 6 and 16 (Sandhu *et al*., 1994, 1996; Reddy *et al*., 1999, 2000). Chromosome 16 is of particular interest because an abundance of evidence points to the presence of multiple tumor suppressor genes on the long arm of 16. Rearrangements of 16q have frequently been documented in a number of human cancers, including breast (Lindblom *et al*., 1993; Cleton-Jansen *et al*., 1994; Tsuda *et al*., 1994; Driouch *et al*., 1997; Hansen *et al*., 1998), ovarian (Hansen *et al*., 2002), prostate (Suzuki *et al*., 1996; Godfrey *et al*., 1997), lung (Sato *et al*., 1998) and hepatocellular carcinomas (Bando *et al*., 2000; Balsara *et al*., 2001). Loss of chromosome 16 was also observed in SV40 immortalized human fibroblasts (Hubbard-Smith *et al*., 1992). Frequent loss of heterozygosity (LOH) of chromosome 16 markers has identified 16q22.1, 16q23.2–q24.1 and 16q24.3 as commonly deleted regions in breast, liver and prostate cancer (Cleton-Jansen *et al*., 2001). Analysis of 21 established breast tumor cell lines also revealed deletions predominantly located at 16q22.1 and 16q24.3 (Callen *et al*., 2002). 16q alterations have been observed in breast tumors with little or no abnormality in the rest of the chromosome complement, implying that they may be early events in breast carcinogenesis. Cytogenetic analysis of E1A-transformed nontumorigenic prostate epithelial cell lines revealed a consistent deletion on 16q (Chin *et al*., 1998), indicating that 16q loss is concomitant with cellular immortalization. A high incidence of 16q LOH observed in ductal carcinoma *in situ* (DCIS), a pre-invasive state of ductal breast cancer, also implicates 16q as an early event in the etiology of breast cancer (Lakhani *et al*., 1995; Radford *et al*., 1995; Vos *et al*., 1999). Frequent LOH observed at three different sites on 16q, associated with many different type of cancers, suggests that it may harbor more than one tumor suppressor gene.

Microcell-mediated transfer of normal chromosome 16 or 16q22-qter restored replicative senescence in immortal cell lines derived from breast, ovarian and prostate tumors and SV40-transformed cells (Reddy *et al*., 1999). While restoration of senescence, following

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chromosome transfer into immortal cells, implies the presence of senescence genes on the donor chromosome, reversion of senescent cells to immortal growth can result from inactivation of senescence gene(s) due to a mutation or deletion. Analysis for the loss of DNA markers, in revertant clones originating from independent senescent microcell hybrids, mapped the SEN16 locus within a 3 cM genetic interval at 16q24.3 (Reddy *et al*., 1999). The precise positional information allowed us to identify candidate Yeast Artificial Chromosome (YAC) clones, which may carry the SEN16 locus. The functional testing of individual YAC clones, located at 16q24.3, led to the identification of a single 360 kb YAC that carries the SEN16 gene (Reddy *et al*., 2000). This paper reports the identification of an 85 kb BAC clone that carries the SEN16 gene, by functional complementation of immortal breast tumor cells.

Results

Construction of a BAC contig at the SEN16 locus

We used two approaches to identify BAC clones from the SEN16 locus: screening of a PCR-ready human BAC library and searching the human genome database. In all, 25 STS markers, previously mapped to the SEN16 locus by deletion analysis (Reddy *et al*., 1999), were used to identify corresponding BAC clones by library screen or database search. A total of 35 BACs identified by these methods were purchased from Invitrogen, CA, USA. These BACs were tested to confirm the expected markers and other adjacent markers. These data allowed us to confirm overlapping BAC clones and assemble a preliminary physical map corresponding to a 3 cM genetic interval between markers D16S520 and D16S413 at 16q24.3. Our preliminary contig map was interrupted with several gaps. Next, BACs flanking each gap were partially sequenced at both ends of the insert. PCR primer pairs from each end sequence (Table 1) were used to rescreen the human BAC library. BAC clones identified in the second screen were tested with BAC end markers as well as with other mapped markers to confirm gap closure. Some overlaps were identified by homology search of BAC end sequences in the genome databases. These data allowed us to complete a detailed physical map for a 1.3 Mb genomic region at 16q24.3, comprised of 17 BAC clones (Figure 1). The relative order of markers and BAC clones was confirmed and updated by comparison with the available draft

Table 1 Primer pairs corresponding to the ends of human genomic inserts in BAC clones

Primer	*Forward*	*Reverse*
143f5.sp6	GACATACGAAGGGCTTCTGG	GGAAATCATTCCGAAGGTGA
143f5.t7	CCCACCCCTTTACTCACTCA	GAAGCCCCCACACTTCATTA
346j21.t7	CGCACGTGCTTCTGATTAAA	AGGAGCCACATGAACTGACC
344a17.t7	GAACGATCATGGCTGGTTTT	CTTGGCTCCTGACCTTTCAC
344a17.sp6	GGCTTTGAGCCCTATCTTCC	CAACTTGCAAACTCCTGCTG
343h9.t7	TGGGACTACAGGCTCACTCC	AAGGCCCAAACTGGATTTA
343h9.sp6	CCAACAGCTACTGAACTAAGTGGA	CATCAGAGTCGCGTGTCATT

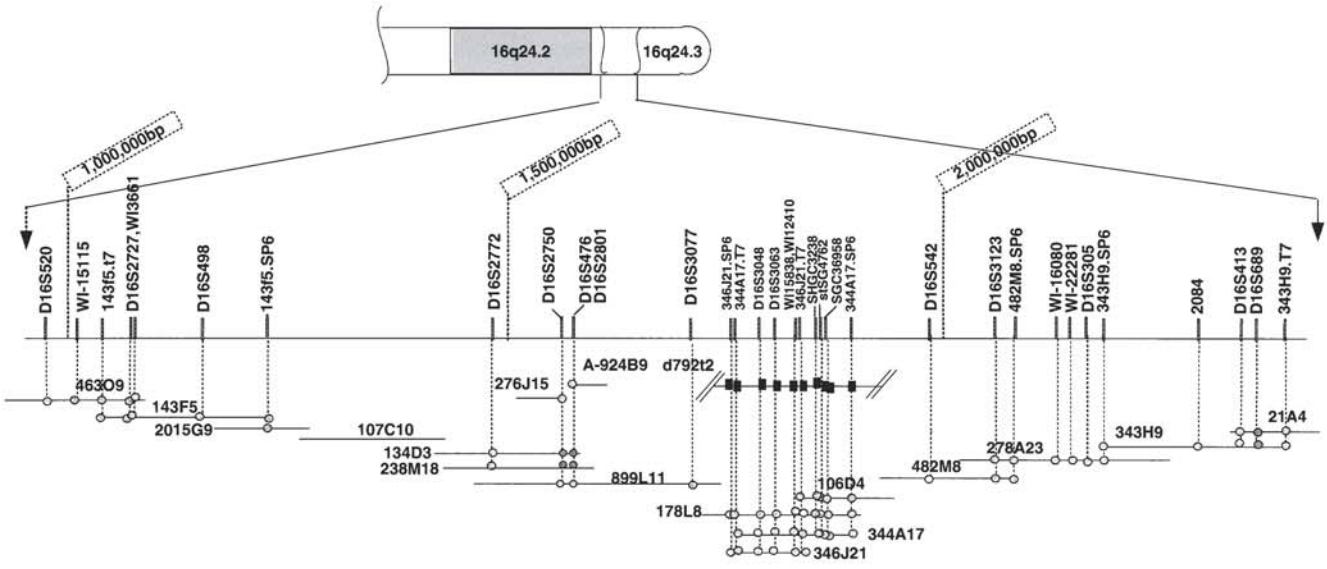

Figure 1 Physical map and BAC contig of chromosome 16 around SEN16, showing markers confirmed by PCR. The distance scale of the map (vertical dotted lines) is based on the draft sequence of the 2.7 Mb human chromosome 16 genomic contig NT_019609

sequence of chromosome 16 (http://www.ncbi.nlm.nih.gov/mapview/maps.cgi). Each BAC insert was sized by pulsed field gel electrophoresis (PFGE) and fingerprinted after digestion with *Hind*III.

Four of the BAC clones in Figure 1 overlay YAC792E1, which was previously shown to induce senescence in immortal breast tumor cell lines (Reddy *et al*, 2000). The 150 kb insert of 344A17 and the slightly larger insert of 178L8 both carry all seven STS markers mapped to the 360 kb insert of YAC792E1. The 85 kb insert of 346J21 carries four markers from the left side of YAC792E1, which are associated with senescence activity (Reddy *et al*., 2000).

Transfer of BACs into immortal cells

To test for SEN16 function in immortal tumor cell lines, BACs were first retrofitted with a *neo* marker to permit selection of transfected cells in G418. BAC retrofitting was accomplished by excising the human insert from the parent vector with *Not*I and recloning into pJMOx166, a BAC vector that carries the *neo* marker. BACs 344A17, 178L8, and 106D4 proved difficult to retrofit because of multiple internal *Not*I restriction sites, but 346J21 has no internal *Not*I sites and was successfully retrofitted. Two BACs from the contig, 924B9 and 276J15, which do not overlap YAC792E1, were chosen as negative controls for functional tests, and were also retrofitted successfully. The integrity of retrofitted BACs was confirmed by PCR for human markers as well as by *Hind*III fingerprinting of the insert (data not shown). The origin of the human inserts in retrofitted 346J21 and 924B9 was tested by FISH. Retrofitted 346J21 showed a single hybridization signal at 16q24.3 (Figure 2), but 924B9 is chimeric and lit up chromosome 13 as well as 16 (data not shown).

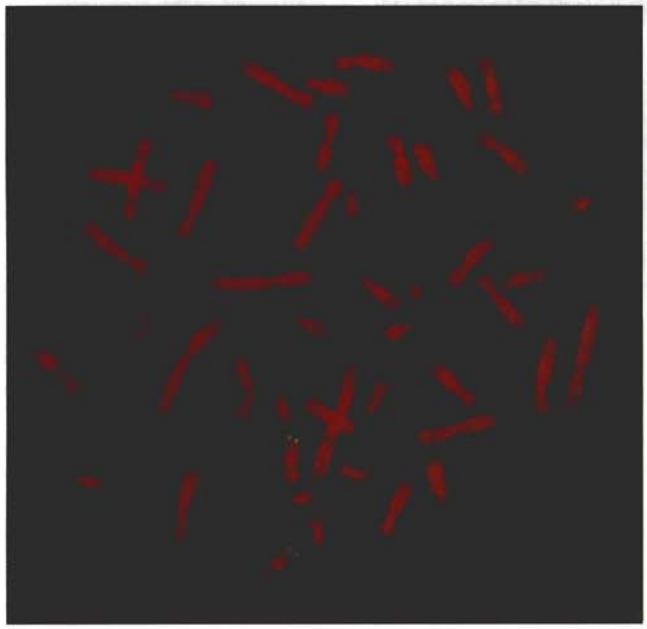

Figure 2 A metaphase spread of normal human chromosomes showing FISH of DNA from retrofitted BAC 346J21 at 16q24.3

Purified retrofitted DNA from BACs 346J21, 924B9, and 276J15 was introduced into human and rodent immortal tumor cell lines using lipofection or electroporation. The empty retrofitting vector was included as an additional negative control. The recipient cell lines for BAC transfer experiments included MCF.7 and MDA-MB468 (human breast tumor cell lines), T98G (human glioblastoma cell line), LA7 (rat mammary tumor cell line), and A9 (mouse connective tissue cell line). Previous studies showed that introduction of a normal human chromosome 16 restores senescence in MCF, MDA-MB468, and LA7 cells, but not in T98G and A9 cells (Reddy *et al*., 1999). BAC transfer colonies were maintained in G418 throughout each experiment, unless otherwise noted. The number and phenotype of colonies recovered from each transfection experiment are recorded in Table 2.

BAC 346J21 restores senescence in rat mammary tumor cells

Transfer of retrofitted BAC 346J21 into LA7 rat mammary tumor cells produced three types of colonies: 12 senescent colonies, eight parental type colonies, and 15 colonies comprised of a mixture of senescent and parental type cells (Table 2). Senescent LA7/346J21 colonies were comprised entirely of enlarged, flattened, vacuolated cells (Figure 3) with an initial doubling time of 30–40 h, which increased progressively until complete growth arrest. At this stage, each colony contained 300–400 cells. After 4 weeks, one of the senescent colonies was stained to confirm the presence of senescence-associated β-galactosidase (SA-β-gal) activity (Figure 3). Half the remaining senescent colonies were harvested by trypsinization and pooled for PCR analysis. Five other senescent colonies were maintained in G418 until they peeled off the plate 1–2 months after plating. Peeled cells from each colony were collected for PCR analysis. DNA from the senescent cells harvested by trypsinization (LA346senpool), and from each of the peeled senescent colonies (e.g. LA346sen1), was positive for the *neo* marker as well as four chromosome 16 markers present on 346J21 (Table 3).

In contrast, cells in parental type colonies were morphologically indistinguishable from the parental LA7 cells and grew with the same 15–18 h doubling time as the parental cells. After 2 weeks, parental colonies were transferred to fresh plates and passaged in G418 for 2 months with no decrease in growth rate. PCR analysis of six of these clones (e.g. LA346par1) confirmed the presence of the *neo* marker, but failed to detect four chromosome 16 markers present on 346J21 (Table 3).

In all, 15 colonies were observed to consist of a mixed population of cells with a senescent morphology, which stained positive for SA-β-gal, interspersed with cells of parental morphology, which stained negative for SA-β-gal. As expected, passaging of mixed clones resulted in progressive dilution of the senescent-type cells. A pooled

Table 2 Number and phenotype of immortal tumor cell lines following BAC transfer into immortal cell lines

BACs	*Cell lines*	*# of expts.*	*Number of colonies*				*Cell division interval (h)*[a]	*Length of survival of senescent colonies*[b] *(weeks)*
			Senescent	*Mixed cell population*	*Parental type*	*Total*		
346J21	LA7	4	12	15	8	35	30–40	4–6
	MCF.7	4	28	38	6	72	90–96	5–7
	MDA-MB468	2	30	—	48[c]	78	90–96	5–7
	T98G	2	0	0	35	35	20–24	imm[d]
	A9	2	0	0	46	46	18–20	imm
924B9	LA7	4	0	0	25	25	15–18	imm
	MCF.7	4	0	0	60	60	20–24	imm
	MDA-MB468	2	0	0	45	45	20–24	imm
	T98G	2	0	0	26	26	20–24	imm
	A9	2	0	0	37	37	18–20	imm
276J15	LA7	4	0	0	30	30	15–18	imm
	MCF.7	4	0	0	39	39	20–24	imm

[a]Determined by counting cells under phase contrast microscope at regular intervals of cell growth. [b]Interval between time of BAC transfer and peeling off of senescent clones. [c]Represents a total of mixed cell population and parental-type cells. [d]Immortal cell population

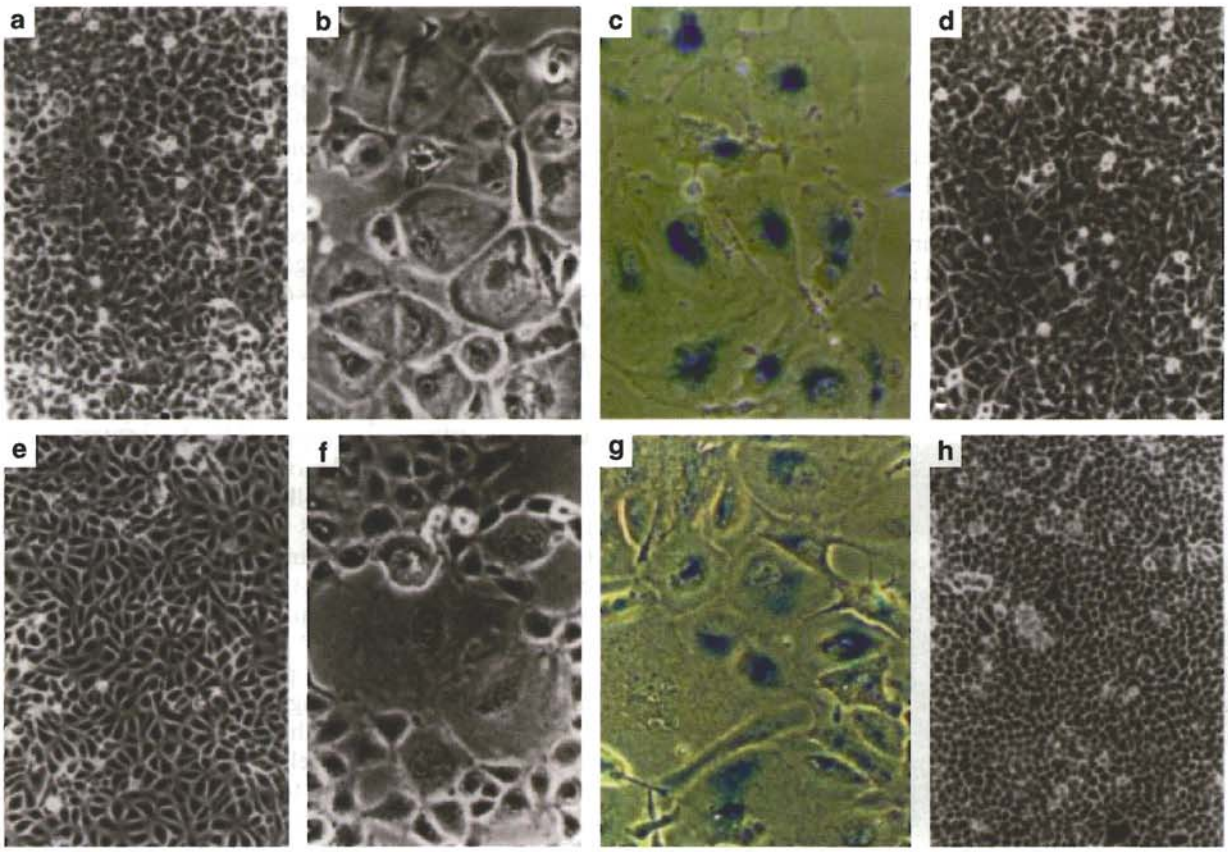

Figure 3 Photomicrographs of human (MCF.7) and rat (LA7) mammary tumor cells and BAC transfer colonies. (**a**) LA7 parental cells; (**b**) a senescent LA7/346J21 colony showing flat, enlarged, and vacuolated cell morphology; (**c**) cells from senescent LA7/346J21 colony stained with SA-β-gal; (**d**) an immortal LA/ 924B9 clone; (**e**) MCF7 parental cells; (**f**) a senescent MCF/346J21 colony showing flat, enlarged and vacuolated cell morphology; (**g**) a senescent MCF.7/346J21colony stained with SA-β-gal; and (**h**) an immortal MCF.7/924B9 clone (photomicrographs taken at the same magnification)

mixed cell population was shown to be positive for the *neo* marker as well as for four chromosome 16 markers located on BAC 346J21. Subcloning of these colonies in G418 gave rise to pure parental clones that retained the *neo* marker but had lost most or all of the chromosome 16 markers (e.g. Table 3, LA346R1 and R2).

Table 3 PCR analysis of BAC346J21 markers in BAC transfer clones of LA7 and MCF.7

Marker	*B346J21*	*B346J21cl5*[a]	*LA7*	*LA346senpool*	*LA346sen1*	*LA346par1*	*LA346R1*	*LA346R2*	*MCF.7*	*MCF346sen1*	*MCF346sen2*
Neo	○	•	○	•	•	•	•	•	○	•	•
D16S3063	•	•	○	•	•	○	•	•	•	NP	NP
D16S3048	•	•	○	•	•	○	○	○	•	NP	NP
WI12410	•	•	○	•	•	○	○	○	•	NP	NP
WI15838	•	•	○	•	•	○	○	○	•	NP	NP

White circles – negative; black circles – positive; NP – nonpolymorphic. [a]Retrofitted BAC346J21

We conclude that transfer and retention of 346J21 induces senescence in immortal LA7 rat mammary tumor cells. G418-resistant parental colonies arise from the retention of incomplete BAC 346J21 DNA following transfer into LA7 cells. Mixed colonies arise from transfer of intact 346J21, followed by reversion due to subsequent loss of part of 346J21 from one or more cells in the colony.

BAC 346J21 restores senescence in human breast tumor cells

Transfer of retrofitted 346J21 into MCF.7 cells also produced three types of colonies. A total of 72 colonies obtained included 28 senescent colonies, six parental type colonies, and 38 mixed colonies. Transfer of retrofitted 346J21 into MDA-MB468 cells gave rise to two types of colonies, which included 30 senescent and 48 parental type colonies (Table 2). Senescent colonies in both human cell lines consisted of enlarged, flattened, vacuolated cells (Figure 3) that grew with an initial doubling time of 90–96 h and ceased to divide after 4–6 weeks. These growth-arrested colonies contained 300–1000 cells that stained positive for SA-β-gal (Figure 3) and peeled off the plates after an additional 3–6 weeks. In contrast, parental-type colonies of MCF.7/346J21 and MDA-MB468/346J21 were indistinguishable from the parental cell line and did not stain for SA-β-gal activity.

Retention of intact 346J21 DNA in MCF.7/346J21 or MDA-MB468/346J21 cells could not be confirmed due to lack of polymorphic differences in the DNA markers between the BAC and the recipient cells, but both parental and senescent colonies tested positive for the retention of *neo* gene, as expected (Table 3). However, when some of the colonies consisting only of senescent cells were transferred to nonselective medium lacking G418, after 4–6 weeks they were overgrown by a segregant cell population that had growth and morphological characteristics similar to parental MCF.7 cells. Parental type segregants were unable to grow when re-cultured in G418 medium, confirming the loss of BAC DNA.

In parallel experiments, we also transferred BACs 924B9 and the vector pJMOx166, into LA7 and MCF.7, and MDA-MB468 and BAC 276J15 into MCF.7 and LA7 cells. These experiments produced only parental-type colonies (Table 2, Figure 3). PCR analysis of parental-type MCF.7/924B9 colonies, for the polymorphic marker D16S476 and for *neo*, showed that the donor BAC 924B9 was retained in each case. Similarly, PCR analysis of LA7/924B9 and LA7/276J15 clones for markers confirmed the retention of the BAC in these colonies (data not shown). These negative controls indicate that the human DNA sequence in 346J21 is responsible for induction of senescence in MCF.7, MDA-MB468 and LA7 cells.

BAC 346J21 has no effect on T98G human glioblastoma or mouse A9 connective tissue cells

Transfer of retrofitted 346J21 into T98G and A9 cells produced 35 and 46 colonies (Table 2). All T98G/346J21 and A9/346J21 clones displayed the parental phenotype, with morphology and growth characteristics indistinguishable from T98G or A9 cells. PCR analysis of parental A9 clones confirmed retention of 346J21 markers. As expected, transfer of 924B9 into T98G and A9 cells also produced only parental colonies.

Discussion

Applying a functional approach, we have identified a BAC, 346J21, that restores a normal cell growth phenotype and restricts replicative potential in human breast and rat mammary tumor cell lines. Starting with an intact chromosome (Reddy *et al.*, 1999, 2000), we progressively narrowed the position of SEN16 to the 85 kb insert of 346J21. Cells that retain the transferred BAC are able to undergo 8–10 rounds of cell division before terminal growth arrest. This is sufficient to observe the formation of colonies carrying the transfected BAC and to test for retention of BAC markers in heterospecific host cells. Growth retardation is accompanied by the appearance of differentiated cell morphology and the senescence-associated β-galactosidase marker. The phenotype observed in 346J21 transfer clones is identical to the phenotype obtained after the transfer of an intact normal chromosome 16 into the same cell lines. BAC 346J21 also shows the same specificity as chromosome 16, restoring senescence in MCF.7, MDA-MB468, and LA7, but not in T98G and A9 cells. Growth arrest in BAC transfer clones is irreversible as long as the BAC DNA is retained by the cells, but is completely reversed upon loss of a part or all of the transfected human DNA sequences, giving rise to revertant or segregant colonies that are indistinguishable from the parental cell line.

The simplest explanation of our complementation data is that 346J21 carries a gene, SEN16, that plays an essential role in a pathway leading to replicative senescence in mammary epithelial cells, which is inactivated on both copies of chromosome 16 in immortal human breast and rat mammary tumor cell lines. We postulate that inactivation of SEN16 is an early event in cancer progression, permitting clonal expansion and accumulation of additional mutations. Chromosome transfer experiments suggest that the same gene may be implicated in the immortalization of ovarian and prostate epithelial cells (Reddy *et al.*, 1999). BAC 346J21 has no effect on some other immortal cell lines such as T98G and A9, suggesting that immortalization of these cell lines involved inactivation of other genes in the same pathway or in a different pathway leading to senescence.

Chromosome 16q has been extensively scrutinized by different methods such as comparative genomic hybridization (CGH), LOH, and sequence analysis in search of tumor suppressor genes (Whitmore *et al.*, 1998a; Powell *et al.*, 2002; Vivienne Watson *et al.*, 2004). High incidence of LOH reported at three different regions of 16q, in several different types of cancers, suggest that more than one tumor suppressor genes may be located on 16q. A number of potential candidate tumor suppressor genes for breast cancer (Bednarek *et al.*, 2000; Kochetkova *et al.*, 2002), prostate cancer (Vivienne Watson *et al.*, 2004), and hepatocellular carcinoma (Saffroy *et al.*, 2002) have been identified by virtue of differential expression or deletion in tumor vs normal tissue.

The BAC mapping and transfer studies reported here, together with previously reported YAC transfer studies (Reddy *et al.*, 2000), establish that SEN16 is distinct from six other candidate tumor suppressor genes that have been reported at16q24 (Figure 4). The WWOX gene, which encodes an oxidoreductase-like protein that may play a role in apoptosis, is underexpressed in many breast cancer cell lines, and upon re-expression inhibits tumorigenicity and anchorage-independent growth; it maps at the fragile site FRA16D between D16S516 and D16S518 about 8 Mb centromeric from 346J21 (Bednarek *et al.*, 2000, 2001). The 17-β-HSD2 gene, which encodes an enzyme involved in estrogen metabolism and is frequently deleted in human hepatocellular carcinoma (Huang *et al.*, 2001), has been mapped between D16S505 and D16S422, approximately 5 Mb centromeric from BAC 346J21. CDH13, which encodes a cadherin-like protein that may be involved in cell surface adhesion and may be either up- or downregulated in tumor cells, maps between D16S422 and D16S402 (Kawakami *et al.*, 1999; Chalmers *et al.*, 2001), about 3.7 Mb centromeric from BAC 346J21. The WFDC1 gene (Larsen *et al.*, 2000), which encodes the secreted ps20 protein and is growth inhibitory in prostate carcinoma cells, maps between D16S402 and D16S3061, about 3 Mb centromeric from BAC 346J21. The CBFA2T3 gene, which encodes a member of the ETO family of proteins that may be involved in transcriptional regulation, is underexpressed in breast cancer cells and upon re-expression inhibits cell growth (Kochetkova *et al.*, 2002); it maps between D16S413 and D16S3026, about 1 Mb telomeric from BAC 346J21. The GAS11 gene, the human homolog of a growth arrest-specific mouse gene, maps near D16S303 close to the telomere (Whitmore *et al.*, 1998b), about 2 Mb telomeric from BAC 346J21. A physical map of these genes relative to 346J21 is given in Figure 4.

Computer analysis of the DNA sequence around 16q24 predicts 10–11 unigene clusters on BAC 178L8 (Powell *et al.*, 2002), several of which are also present on 346J21 which overlays 178L8 (see Figure 1). Detailed characterization of transcripts from BAC 346J21 will allow us to confirm the identity of SEN16 and explore its integrity and expression in tumor cells and in normal cells as they approach senescence.

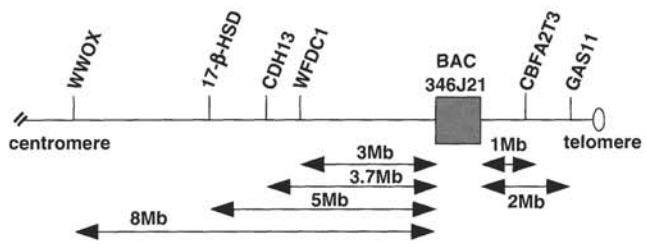

Figure 4 Physical map of 16q24, showing approximate distances between reported candidate tumor suppressor genes and BAC 346J21. Distances are based on published mapping data and sequence information retrieved from http://www.ncbi.nlm.nih.gov/

Materials and methods

Cell lines and culture conditions

Cell lines used in these studies included MCF.7 and MDA-MB468 (human breast tumor), T98G (human brain tumor), LA7 (rat mammary tumor), and mouse A9 cells. The normal human cell line GM03468A (Human Mutant Cell Repository, Camden, NJ, USA) was used for the phenotypic comparison of replicative senescence. All cell lines were routinely cultured at 37°C in a 7.5% CO_2/air incubator in DF12 medium supplemented with 10% fetal bovine serum. Base medium was supplemented with G418 (400 μg/ml) for the selection and propagation of BAC transfer colonies. Colonies were refed at 3-day intervals.

Identification of BAC clones

PCR ready DNA pools of a human BAC library, comprised of 150 K clones (Invitrogen, CA, USA), were screened with primers for 25 STS markers (shown in Figure 1) mapped at the SEN16 locus (Reddy *et al.*, 1999). End sequences obtained from some BACs were used either for rescreening of the BAC library, using the PCR primers listed in Table 1, or for a homology search of the human genome database. The BAC clones thus identified were purchased from Invitrogen, CA, USA. Individual BAC clones were propagated in LB medium containing 12.5 μg/ml chloramphenicol and subcloned to generate single-cell clonal populations.

Retrofitting of BAC clones

BAC clones were retrofitted to incorporate a *neo* gene for the selection of mammalian cells in G418. Retrofitting was carried out using the procedure and the plasmid described by Mejia and Monaco (1997). Briefly, the human DNA insert in a BAC clone was released by digestion with *Not*I and fractionated by PFGE. Human DNA purified from the agarose gels was then ligated with *Not*1 linearized and dephosphorylated pJMOx166 vector DNA (Mejia and Monaco, 1997). This vector carries a *cat* gene to select for chloramphenicol resistance in *Escherichia coli* cells and a *neo* gene for selection of mammalian cells in G418. Ligated DNA was transformed into *E. coli* DH10B cells and the transformation mix was plated on LB medium containing 30 μg/ml kanamycin and 20 μg/ml chloramphenicol. The resulting transformant clones were analysed for the presence of STS markers and by PFGE to confirm the presence and the size of the human insert.

Transfer of BAC DNA into immortal tumor cells

DNA, for each retrofitted BAC, was isolated from 500 ml cultures by alkaline lysis and purified through Qiagen 500-ml tip columns. Purified BAC DNA was introduced into immortal recipient cells using lipofection or electroporation methods. For lipofection, recipient cells (5×10^5/100 mm plate) were seeded in 100 mm tissue culture dishes, 24 h prior to transfection. BAC DNA (10 μg) was mixed with 300 μl of lipotaxi reagent (Stratagene) and added to the medium in a monolayer of recipient cells. After 12 h of incubation, the medium was replaced with fresh medium. Following 24 h of growth in nonselective medium, cells were split into two plates and fed with medium containing G418 (400 μg/ml). Colonies were either isolated individually in separate plates or followed in the parent plates. This protocol worked efficiently on MCF.7 cells but not LA7 cells. For electroporation, 5 μg of BAC DNA was mixed with 1×10^7 recipient cells in a 0.4 cm gap cuvette and pulsed at 300 V with 500 or 960 μF capacitance. Electroporated cells were plated in 100 mm tissue culture dishes in nonselective medium. After 24 h of expression time, the medium was replaced with selection medium containing G418.

Analysis of BAC transfer colonies

G418-resistant colonies appeared after 1–2 weeks. Thereafter, each colony was examined at regular intervals under a phase contrast microscope to assess colony morphology and to monitor growth. Cell doubling time was determined by counting cells under the microscope or in photomicrographs. Individual colonies were stained for senescence-specific acidic β-galactosidase using published procedure (Dimri *et al*., 1995).

FISH

Biotin or digoxigenin-labeled probes made from BAC clones were hybridized to human metaphase chromosomes derived from PHA-stimulated peripheral blood cultures. The conditions of hybridizations, signal detection, digital-image acquisition, processing, and analysis, as well as the procedure for direct visualization of fluorescent signal to labeled chromosomes, were carried out as described previously (Zimonjic *et al*., 1995).

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